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14. ABSTRACT This proposal is to determine if radiation affects presentation of prostate specific antigen (PSA) through endogenous and exogenous pathways by dendritic cells (DCs) and to devise strategies to enhance the manner in which radiation-induced cell death is translated into the generation of tumor-specific immunity so as to achieve the best therapeutic outcome from radiation therapy. From the conventional point of view, radiotherapy is usually related to cell killing. However, our hypothesis is that radiation is more than just depleting cells. It also influences functional antigen presentation by DCs without killing them. We chose PSA as antigen for this study, but because of the high risk nature of the experiments and the high PSA expression levels in prostate cancer patients, we have also explored the possibility of monitoring the immune responses to the antigen survivin in prostate cancer. Our approach to translate radiation-induced immunosuppression into beneficial tumor immunogenicity focuses on IL-3 and/or GM-CSF treatment. Our studies on combined treatment of radiotherapy and IL-3, and on the effects of radiation on PSA presentation by DCs are underway and are presented in this Progress Report, along with other milestone that have been attained.					
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## Table of Contents

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4</b>
<b>Key Research Accomplishments.....</b>	<b>9</b>
<b>Reportable Outcomes.....</b>	<b>9</b>
<b>Conclusions.....</b>	<b>10</b>
<b>References.....</b>	<b>n/a</b>
<b>Appendices.....</b>	<b>10</b>

## Introduction

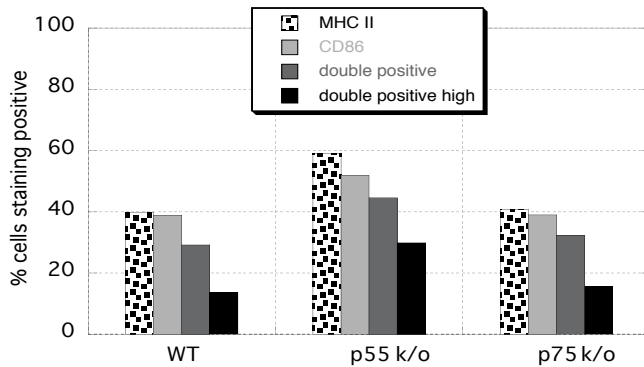
The immunosuppressive effects of ionizing radiation are generally considered to be due to killing of radiosensitive lymphocytes. However, we discover another and novel mechanism of radiation-induced immunosuppression, which is the inability of dendritic cells (DCs) to process antigens. We believe that radiation does this by inhibiting proteasome function and/or the expression of cytokines and related molecules by DCs. To overcome this radiation-induced immunosuppression, we plan to investigate the combined effects of radiation and cytokines that we have shown to increase tumor immunogenicity, specifically IL-3 and/or GM-CSF. The overall goal in this study is to translate radiation-induced tumor cell killing into generation of tumor immunity in the hope of optimizing therapy for localized and disseminated prostate cancer. The aims are unaltered from the original submission.

## Body

The statement of work for this year covers experiments aimed at studying the effects of radiation on the immune responses to DCs with adenovirally delivered PSA (AdV-PSA), on radiation-induced cytokines, cytokine receptors, and proteasome structures, and the development of a treatment regimen with AdV-IL-3 combined with radiation.

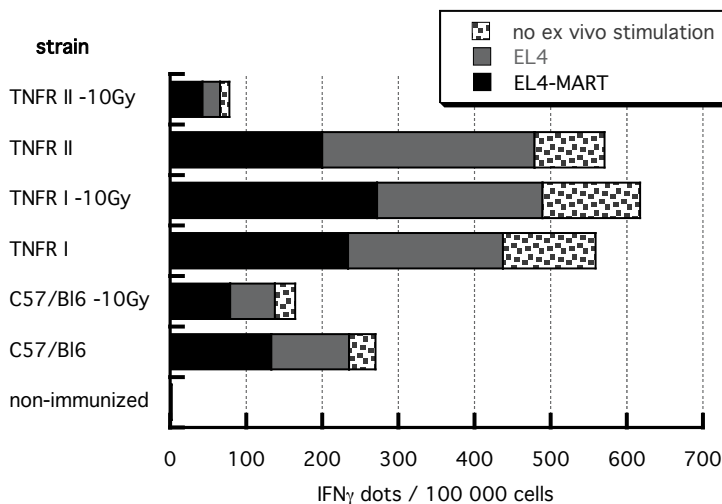
We have spent this year establishing a prostate cancer mouse model, preparing vectors, cell lines and reagents for future experiments and refining our experiment approach. Our AdV-PSA has been constructed, as has our murine prostate cancer cell line expressing PSA and we have initiated our murine studies using these reagents. While these were being developed, we explored a number of the specific hypotheses in the proposal.

Our hypothesis is that radiation affects DC function. One hypothesis was that radiation-induced cytokines or receptors might mediate this form of radiation-induced immune suppression. To test this we screened media from irradiated and non-irradiated DCs for 31 cytokines and receptors. We discovered that the levels of the immunosuppressive cytokines, IL-10 and TGF- $\beta$ , were not enhanced after 10 Gy irradiation (see last report), indicating that these immunosuppressive cytokines were not responsible for radiation-induced DC malfunction. However, interestingly, DCs released high levels of TNF receptor I and II (TNFR I and TNFR II) following irradiation. Shedding of TNFR from the cell surface occurs constitutively but is also induced during immune responses. It is involved in limiting innate immune responses and may also be important for Th1 polarization. In an adaptive immune setting, a neutralizing antibody to TNFR has been shown to inhibit IL-12 release by DCs and, as a consequence, IFN $\gamma$  production by T cells during an allogenic MLR (Becher et al). Since TNFR II engagement is important for co-stimulation of T cells, by releasing TNFR, DCs may limit the extent of vital co-stimulation of T cells to which they are presenting antigen. We are therefore following up our findings on radiation-induced shedding of TNFRs by DCs as a possible mechanism for their diminished ability to induce T cell priming in vivo. We are testing this hypothesis using two different knockout mice, TNFR I (p55 $^{-/-}$ ) and TNFR II (p75 $^{-/-}$ ), both on the C57/BL6 background.



**Fig. 1.** Maturation status of BMDCs. DCs were grown in the presence of IL-4 and GM-CSF and harvested on day 9. Cells were double stained with antibodies for MHC II and CD86 and analyzed by flow cytometry

typically associated with DCs. However, according to maturation markers on the cell surface, bone marrow-derived DCs (BMDCs) from these TNFR I ko mice not only had a higher proportion of cells staining positive for CD86, MHC II, and both molecules, but also had higher levels of these surface markers than WT BM-DCs (Fig. 1). BMDCs from TNFR II ko appeared to have somewhat similar expression of markers to the WT BMDCs and similar morphology. We concluded that loss of TNFR I alters the morphology of DCs and enhances their expression of the co-accessory markers that are associated with superior antigen presentation.



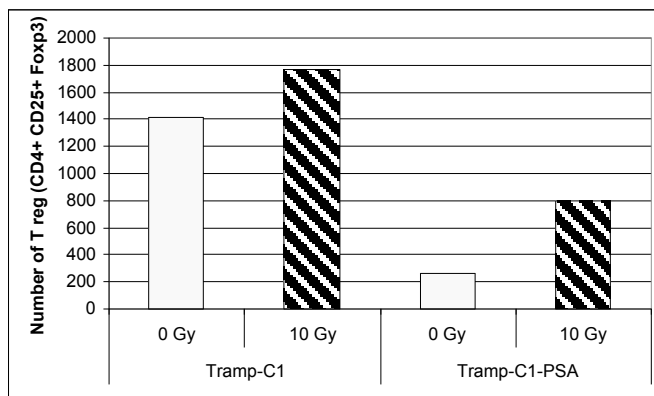
**Fig. 2.** Antigen presentation by BMDCs. DCs were grown from bone marrow cultures of WT, p55/- or p75/- in the presence of IL-4 and GM-CSF. On the day of harvest, DCs were irradiated with 10Gy or not and transduced with AdMART prior to injection into WT C57/Bl6 mice ( $5 \times 10^5$  DCs/mouse). 7 days later splenocytes were harvested, re-stimulated ex vivo with either EL4 or EL4-MART system or left non-stimulated. T cell cultures were analyzed by ELISPOT to measure IFN $\gamma$  production by activated T cells.

We then examined whether these phenotypic differences between DCs from WT mice and TNFR ko mice were reflected differences in their functional status. Initially, we chose the AdV-MART antigen to perform these studies because this was our original “test” system for the radiation effects on DCs and because we had more experience with this than with PSA, although similar experiments with the PSA system are planned. Overall, BM-DCs from TNFR ko were superior to WT BM-DCs in terms of antigen presentation, as confirmed by ELISPOT (Fig. 2), indicating that these receptors generally act as a ‘brake’ on immunity, as has been suggested previously. With time it emerged that this effect was robust and consistent, in particular in the case of the TNFR I ko DCs (not shown). The major ‘brake’ on the development of immunity by DCs seems therefore

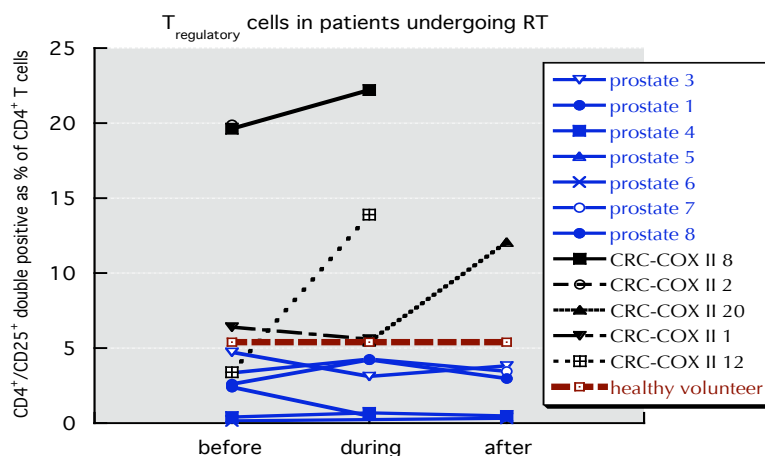
to be TNFR I. It is possible that it inhibits their phenotypic maturation. This was somewhat surprising since it has been reported in the literature that DCs respond to autocrine TNF during maturation, even more so through TNFR I than TNFR II.

Regarding the response to radiation, it appears that DCs from TNFR II ko mice respond to 10 Gy in a similar fashion to those from WT mice, in that they have a decreased ability to generate T cell responses to antigen following injection. In contrast, 10 Gy irradiation did not suppress this ability in TNFR I ko DCs. These experiments suggest that TNFR I shedding following irradiation may be responsible for radiation-induced inhibition of DC function. Furthermore, they suggest a means of circumventing this effect through the use of drugs that inhibit shedding or antibodies to TNFR II that might stimulate DC maturation and bias the response towards radiation resistance. Further analysis will involve these approaches and the use of double knockouts, which will allow discrimination between the positive and negative effects of these molecules.

We have been addressing the consequences of negative regulation of the immune system by irradiated DCs at another level. It would seem possible that irradiated DCs generate tolerance rather than immunity, and in the last funding period, we presented data to this effect that is currently being prepared for publication. Because of this data, we have been examining whether one of the outcomes of radiation-induced immune suppression is an increase in CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T<sub>reg</sub>) that would suppress the generation of effector T cells. As shown in Fig. 4, the number of T<sub>reg</sub> increased after 10 Gy radiation in vivo both in Tramp-C1 and Tramp-C1-PSA tumors. These data clearly indicate that we will have to take T<sub>reg</sub>s into account in our experimental systems in the future, since they could be the effector mechanism that is generated by irradiated DCs in vivo.



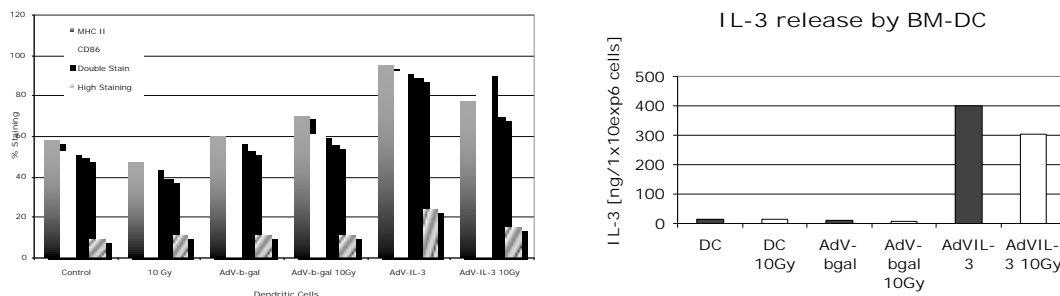
**Fig. 4.** The number of regulatory T cells was analyzed by flow cytometry. Splenocytes of TRAMP-C1 and Tramp-C1-PSA bearing mice were extracted 72 hours after 10 Gy radiation and T<sub>reg</sub>s were co-stained with CD4, CD25 and Foxp3 antibodies.



**Fig. 5.** Levels of T regulatory cells in peripheral blood of patients with prostate cancer or colorectal cancer. Blood was taken before, during and after radiotherapy and analyzed for CD4/CD25 positive T cells by flow cytometry.

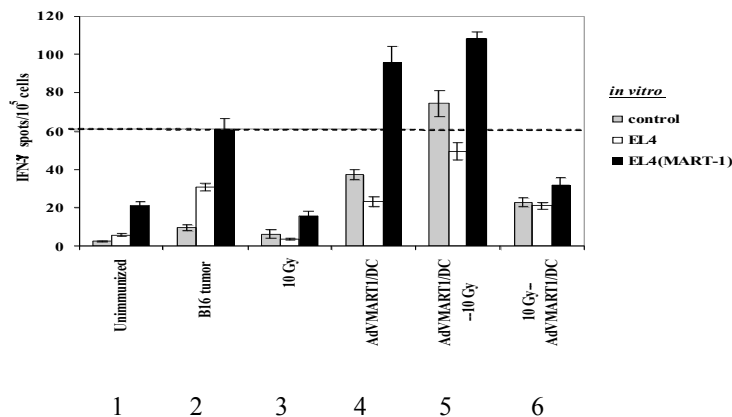
Although not part of this proposal, because of our concerns about  $T_{reg}$ s following irradiation, we undertook to study whether or not there were significant changes in  $T_{reg}$  levels in prostate cancer patients receiving radiation treatment.  $T_{reg}$ s have been found at high levels in many cancers, >5% of CD4+ T cells, but they seemed low in patients with prostate cancer and did not increase following radiation therapy (Fig. 5). The low levels may be due to the relatively localized disease in this patient cohort, but it did reassure us that our attempts at modulating immunity in prostate cancer would not have to overcome the effects of high levels of these cells.

Utilizing IL-3 is one of our proposed approaches to overcome radiation-induced immunosuppression. We started with examining the effects of AdV-IL-3 combined with radiation on co-stimulatory molecules in DCs. As can be seen in Fig. 6 (left), the expression of MHC class II and CD86 was upregulated 4 days after transduction of DCs with AdV-IL-3. In fact, these data may underestimate the effect of IL-3 since there was a subpopulation that had uniquely high CD86 levels after AdV-IL-3 transduction. IL-3 expression was confirmed by ELISA assay (Fig. 6 (right)). The data suggest that IL-3-transduced DCs might have better ability to present antigen. There was no suggestion that these cells would be affected more by irradiation. The experiments to test their functional ability with and without irradiation are underway.



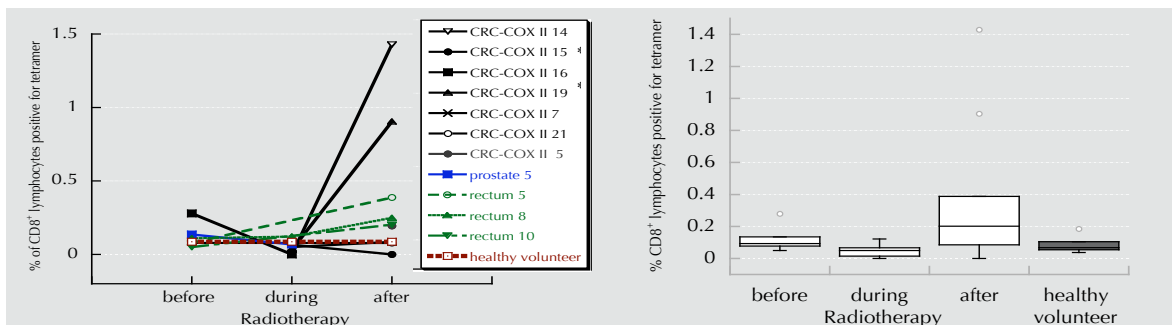
**Fig. 6.** Co-stimulatory molecules of AdV-IL-3 transduced DC by flow cytometric analysis 24 hr after radiation. DCs were transduced with AdV-IL-3 on day 5 and irradiated with 10 Gy on day 8 (**left**). The level of IL-3 expressed in AdV-IL3 transduced DCs was measured by ELISA (**right**).

While constructing AdV-PSA, we initiated a pilot study to explore the possibility that in vivo irradiation may block local DCs ability to present antigens and ways to prevent this by injection of antigen-laden DCs, with an emphasis on the timing of DC administration. As can be seen in Fig. 7, splenic responses could be detected in control mice with B16 tumors (group 2). Tumor irradiation alone decreased the responses (group 3). Intratumoral AdVMART1/DC injection enhanced splenic T cell responses (group 4) and this was not affected and may have been slightly enhanced by irradiation given one day later (group 5). In contrast, local tumor irradiation prior to AdVMART1/DC injection inhibited the generation of splenic T cell responses (group 6). This suggests that irradiation of tumor decreases immunity and blocks its generation, possibly through induction of immune tolerance. This has important implications for the proposed study in this grant, which will explore the use of AdV-PSA and AdV-IL-3 as means for immunizing mice and enhancing the effects of radiation therapy that will be performed in the next grant period.



**Fig. 7.** IFN- $\gamma$  expression measured by ELISPOT assay. AdvMART1/DCs were injected intratumorally into B16 tumors and the tumors were irradiated with 10 Gy one day later. Another group of tumors was irradiated (10 Gy) prior to immunization with  $5 \times 10^5$  intratumoral AdvMART1/DC.

As mentioned in the last report, because of the high risk of the proposed experiment and high background of natural PSA level in prostate cancer patient, we chose to investigate survivin, which is over expressed in many prostate cancers, as a potential back-up antigen. We have incorporated a pilot prostate study into a larger effort that involves collaboration with other labs in Europe. As can be seen in Fig. 8, lymphocytes from patients with prostate and colon carcinoma, taken before, during and after presurgical chemoradiotherapy were tested for the level of CD8<sup>+</sup> T cells which recognize the tumor-associated antigen survivin by tetramer assay. We were able to detect survivin-specific CD8<sup>+</sup> T cells in some patients. These tumor-specific T cells appeared to accumulate after completion of radiation therapy in some patients (abstract attached). These experiments will help guide those in this proposal while not being part of the specific aims of the grant.



**Fig. 8.** Analysis of tumor-specific CD8<sup>+</sup> T cells in peripheral blood of 11 patients with different cancers undergoing radiotherapy. Patients were confirmed to be HLA-A2 positive prior to staining with tetramers presenting the survivin peptide LMLGEFLKL. Cells were co-stained for CD8<sup>+</sup> and non-viable cells were excluded on grounds of high 7-AAD up-take. One healthy volunteer served as control throughout. Data shown as % CD8<sup>+</sup> T cells positive for tetramer **a.** of individual patients and **b.** as overall time course. Colorectal cancer patients (CRC-COX II) were part of a clinical study using radiation treatment in conjunction with a COX II inhibitor. Patients that received placebo are indicated with \*.



### Key Research Accomplishments

1. Study of the effects of radiation on DC co-accessory molecules--completed.
2. Study of the effects of radiation on proteasome structure and function--completed.
3. Study of the effects of radiation on different DC subpopulations--completed.
4. Study of the effects of IL-3 combined with radiation on DC function--50% completed.
5. Development of an AdV-PSA vector--completed.
6. Development of a survivin system--80% completed.

### Reportable Outcomes – Manuscripts and Abstracts

Pajonk, F., A. van Ophoven, C. Weissenberger and W.H. McBride: The Proteasome inhibitor MG-132 sensitizes PC-3 prostate cancer cells to ionizing radiation by a DNA-PK-independent mechanism. Biomedcentral Cancer 5:76, 2005.

Pervan, M., K.S. Iwamoto and W.H. McBride: Proteasome structures affected by ionizing radiation. Molecular Cancer Research 3:381-390, 2005

Demaria, S., N. Bhardwaj, W.H. McBride and S.C. Formenti: Combing radiotherapy and immunotherapy: a revived partnership. Int. J. Radiat. Oncol. Bio. Phys. 63:655-666, 2005.

Tsai, C-H., J-H. Hong, K-F. Hsieh, H-W. Hsiao, W-L. Chuang, C-C. Lee, W.H. McBride, and C-S. Chiang: Tetracycline-regulated intratumoral expression of interleukin-3 enhances the efficacy of radiation therapy for murine prostate cancer. Submitted, 2006.

Schaue, D., Y. Liao, B. Comin-Anduix, A. Ribas, D.C. Altieri, A. Debucquoy, K. Haustermans and W.H. McBride: The Effect of Radiation Therapy on Tumor-Specific Immune Responses. In: Abstracts of papers for the 52<sup>nd</sup> Annual Meeting of the Radiation Research Society, Denver, Colorado, 2004.

### Reportable Outcomes – Presentations

4/27/05

Department of Radiation Oncology  
New York University School of Medicine, New York, NY  
Invited Lecture: “Sense of Danger from Radiation”

10/6-10/8/05

19<sup>th</sup> Meeting of European Macrophages and Dendritic Cell Society, Amsterdam, the Netherlands

Abstract presentation: “Radiation Affects Antigen Processing by Dendritic Cells – A Novel Form of Immune Suppression”

### Reportable Outcomes – Presentations (cont.)

11/16/05

Massey Cancer Center, Virginia Commonwealth University, Richmond, VA

Invited speaker: “The Proteasome in Cancer biology and Therapy”

### Conclusions

This has been a year of refinement of approach and development of more reagents. We have made important advances in the elucidation of the mechanism underlying the effects of irradiation on DC function through examining the roles of TNFR I and II in DC maturation in vitro. We have also examined in some detail the effects of IL-3 expression on DC maturation and function and are greatly encouraged that this approach will reverse radiation-induced immune suppression and allow tumor irradiation to overcome immune suppression. We have started investigating the possibility that local DC irradiation might generate T<sub>reg</sub> cells that would inhibit immunity. This is a concern in animal models, but may not be clinically as prostate cancer patients do not seem to have high levels of these cells, at least in early stage disease, but this still remains a clinical concern for the future. We have had no changes in the directions of the proposed work in the grant, although we have set up a new system using survivin as a tumor antigen in case the high natural level of PSA in prostate cancer patients proves to be a problem. We feel that although we have made progress on many fronts, we are only now in a position to finally bring these efforts together in a truly meaningful way. We are encouraged however that nationally we have been instrumental in enthusing clinicians in the concept of combining radiation therapy with immunotherapy and there is a real desire to move this approach into the clinic.

### Appendices

One published manuscript and one abstract are in the appendix.

Schaue, D., Y. Liao, B. Comin-Anduix, A. Ribas, D.C. Altieri, A. Debucquoy, K. Haustermans and W.H. McBride: The Effect of Radiation Therapy on Tumor-Specific Immune Responses. In: Abstracts of papers for the 52<sup>nd</sup> Annual Meeting of the Radiation Research Society, Denver, Colorado, 2004.

Becher, B., M. Blain, P.S. Giacomini, and J.P. Antel: Inhibition of Th1 Polarization by Soluble TNF Receptors is Dependent on Antigen-Presenting Cell-Derived IL-12. J. Immunol. 162:684-688, 1999.

## **The Effect of Radiation Therapy on Tumor-Specific Immune Responses**

In: Abstracts of Papers for the 52nd Annual Meeting of the Radiation Research Society, Denver, CO, 2004.

Dörthe Schae, Yu-Pei Liao, Begonya Comin-Anduix, Antoni Ribas, Annelies Debucquoy, Karin Haustermans, and William H. McBride

We have shown in a mouse B16 melanoma tumor model that local tumor irradiation depresses tumor-specific systemic immunity. We have also shown that in vitro irradiation of dendritic cells affects their function, effectively blocking tumor antigen processing and their ability to generate protective CD8+ T cell anti-tumor responses. Indeed, irradiated dendritic cells actually suppress the generation of responses. The overall aim of this study is to determine if irradiation of human cancer also results in a loss of tumor-specific T cell reactivity.

Lymphocytes from patients with prostate and colon carcinoma taken before, during and after presurgical chemoradiotherapy were tested for the level of CD8+ T cells that recognize the tumor-associated antigen survivin. Survivin has been shown to be over-expressed in many cancers samples. We have attempted to enumerate the number of CD8+ cells that recognize that specific immunodominant survivin-associated antigenic peptide in the context of HLA-A2.1 using tetramer analysis by flow cytometry. In addition, all patient samples were examined for the number of CD4+, CD25+, Foxp3+ T regulatory cells in the circulation since we hypothesized that these would also change following treatment and might be a cause of radiation-induced immune suppression. Some of the patients with colon carcinoma were part of a clinical trial where they received COX-2 inhibitor as a radiosensitizer to determine if this would enhance tumor-specific immunity and influence the generation of T regulatory cells.

The long term basis for this study is the notion that if radiation-induced tumor cell kill were to be better translated into the generation of tumor-specific immunity, local cure rates might be increased and micrometastases inhibited. This study will lay the basis for such future investigations aimed at converting this state of radiation-induced immune suppression into a more positive anti-tumor immune response.

Key words: tumor-specific T cells, T regulatory cells, survivin, tetramer

# Inhibition of Th1 Polarization by Soluble TNF Receptor Is Dependent on Antigen-Presenting Cell-Derived IL-12<sup>1</sup>

Burkhard Becher,<sup>2</sup> Manon Blain, Paul S. Giacomini, and Jack P. Antel

Th1-polarized CD4<sup>+</sup> T cells are considered central to the development of a number of target-directed autoimmune disorders including multiple sclerosis. The APC-derived cytokine IL-12 is a potent inducer of Th1 polarization in T cells. Inhibition of IL-12 in vivo blocks the development of experimental allergic encephalomyelitis, the animal model for multiple sclerosis. Based on previous work that suggests that the production of IL-12 by activated human central nervous system-derived microglia is regulated by autocrine TNF- $\alpha$ , we wanted to determine whether inhibition of TNF could induce a reduction of Th1 responses by its impact on systemic APCs. We found that soluble TNFR p75-IgG fusion protein (TNFR:Fc) inhibited production of IFN- $\gamma$  by allo-Ag-activated blood-derived human CD4 T cells. We documented reduced IL-12 p70 production by APCs in the MLR. By adding back recombinant IL-12, we could rescue IFN- $\gamma$  production, indicating that TNFR:Fc acts on APC-derived IL-12. Consistent with an inhibition of the Th1 polarization, we found a decreased expression of IL-12R- $\beta_2$  subunit on the T cells. Furthermore, the capacity of T cells to secrete IFN- $\gamma$  upon restimulation when previously treated with TNFR:Fc is impaired, whereas IL-2 secretion was not altered. Our results define a TNF-dependent cytokine network that favors development of Th1 immune responses. *The Journal of Immunology*, 1999, 162: 684–688.

CD4<sup>+</sup> Th cells can be divided into Th1 and Th2 cells by their polarized expression of cytokines (1). Th1 cells predominantly produce IFN- $\gamma$  whereas Th2 cells produce IL-4 and IL-10. Proinflammatory Th1 T cells are considered central to the development of target-directed autoimmune inflammatory disorders including multiple sclerosis (MS)<sup>3</sup> and rheumatoid arthritis (RA) (2, 3). In the animal model of MS, experimental autoimmune encephalomyelitis (EAE), T cells recovered from the inflamed central nervous system (CNS) are predominantly Th1-polarized cells (4). In passive transfer experiments, EAE-inducing encephalitogenic T cell lines or clones are Th1 polarized (5, 6). Th1 cells have an advantage in crossing an endothelial barrier by binding to P- and E-selectin (7). Simultaneous administration of Th2 cytokines (IL-10, IL-4) inhibits disease development (8–10).

The APC-derived cytokine IL-12 is a potent activator of the Th1 phenotype in T cells. In contrast to the Th1 cytokines IFN- $\gamma$  and TNF, the APC-derived cytokine IL-12 is crucial for the induction of EAE (11–13). Th1 cells express higher levels of IL-12R  $\beta_2$  subunit, resulting in higher responsiveness to IL-12 compared with Th2 cells (14). IL-12 production by APC is itself tightly regulated (15, 16). IL-12 production in dendritic cells can be induced by cell to cell contact with activated (CD154<sup>+</sup>) T cells (17), and IFN- $\gamma$  can further enhance IL-12 secretion. Our previous in vitro studies

using human adult CNS derived microglial cells as a source of IL-12 indicated that TNF- $\alpha$  regulates IL-12 production via an autocrine network (18). We found that LPS-stimulated human microglial cells produce TNF- $\alpha$  before IL-12 and that administration of TNFR:Fc or anti-TNF Abs significantly inhibits IL-12 production. These observations indicate that TNF should be considered among cytokines that can drive Th1 responses.

EAE can be induced by active systemic immunization or passive T cell transfer, but not by direct intrathecal administration of myelin reactive cells, indicating a role of systemic immune regulation in the disease process and a rationale for systemic therapeutic intervention. TNFR:Fc and anti-TNF Abs, given systemically, have been used to ablate the development of EAE and have been used in clinical trials for RA and MS (7, 19, 20). Therapeutic effects in EAE were attributed to blocking the effector functions of TNF and lymphotoxin (LT); the effect on immunoregulatory functions was not considered (19). Monthly administration of a TNFR-Ig fusion protein containing the 50-kDa TNFR has been shown to be clinically efficacious in the treatment of RA (21). In contrast, a clinical trial for MS, in which TNFR:Fc was also used in the form of monthly systemic injections, was prematurely halted due to concerns regarding adverse impacts on the disease (22). The mechanism of action (or lack thereof) in these clinical conditions was not established.

We wished to establish whether TNFR:Fc would inhibit IL-12 production by systemic APCs and whether there was an associated reduction in Th1 (IFN- $\gamma$ ) cytokine production by T cells responding to these APCs. For our studies, we determined the effects of TNFR:Fc in an MLR assay, as this system serves as an in vitro model for cell-mediated immunity. Establishing the in vitro effects on immune reactivity of inhibiting TNF/LT should help to predict what effects might be expected by systemic TNF/LT-directed therapies involving molecules that may have complicated in vivo pharmacokinetics.

## Materials and Methods

### Reagents

IFN- $\gamma$  and TNF- $\alpha$  were obtained from Genzyme (Cambridge, MA), IL-12 and anti-IL-12 mAbs from R&D Systems (Minneapolis, MN), shu-TNFR was a generous gift from A. Troutt (Immunex, Seattle, WA).

Neuroimmunology Unit, Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada

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<sup>1</sup> B.B. has a fellowship award from the German Academic Exchange Service (DAAD/HSPH). This work has been supported by the Canadian Multiple Sclerosis Society and the London Life Award.

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<sup>3</sup> Abbreviations used in this paper: MS, multiple sclerosis; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; RA, rheumatoid arthritis; TNFR:Fc, soluble human p75 TNF receptor-IgG:Fc fusion protein; LT, lymphotoxin; LDH, lactate dehydrogenase.

### Isolation of peripheral blood-derived cells

PBMC were isolated from healthy adult volunteer donors by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Baie D'Urfe, Canada). For the isolation of enriched APCs, the PBMC were washed twice with PBS and cultured for 1 h in RPMI 1640 medium (Life Technologies, Burlington, ON, Canada) supplemented with 10% FCS, 2.5 mg/ml penicillin, 2.5 mg/ml streptomycin, and 2 mM glutamine (all from Life Technologies) in 75-cm<sup>2</sup> tissue culture flasks (Falcon, VWR Scientific, Montreal, Canada). The nonadherent cells were removed by gentle shaking. The adherent cells consisted of 95% HLA-DR/B7-2 positive monocytes.

CD4<sup>+</sup> T cells were isolated from PBMC using anti-CD4 mAbs conjugated to magnetic beads (Dynal, Great Neck, NY). The beads were detached from the cells after isolation following the supplied protocol. The cells were then washed with PBS and their purity was of  $\geq 96\%$  as assessed by flow cytometry (23).

### Semiquantitative PCR analysis

Total RNA was isolated using TRIzol Reagent (Life Technologies). To transcribe into cDNA, 3  $\mu$ g RNA, 3.3 mM random hexamer primers (Boehringer Mannheim, Mannheim, Germany), reverse transcriptase buffer, 3 mM dNTPs, 400 U Maloney murine leukemia virus reverse transcriptase (all from Life Technologies), 0.6  $\mu$ l RNA guard, and 3 mM DTT (both from Pharmacia) were added to a total volume of 32  $\mu$ l. The reaction mixture was incubated for 1 h at 42°C followed by a 10-min incubation at 75°C. Primers used for PCR were obtained from Life Technologies and had the following sequences: IL-12R  $\beta_2$  forward, 5'-ACAGGACACACCTCCTGGAC-3'; reverse, 5'-AGAGGGACCTGTGTGTACAC-3'; and  $\beta$ -actin forward, 5'-ATGCCATCCTGCGTCTGGACCTGGC-3'; reverse, 5'-AGCATTGCGGTGCACGATGGAGGG-3'. The primers for IL-12R  $\beta_2$  and  $\beta$ -actin were constructed to generate fragments of 281 bp and 378 bp, respectively. cDNA 200 ng was added to the reaction mixture containing PCR buffer, 0.2 mM dNTPs (Life Technologies), 50 pMol of either primer set for IL-12R  $\beta_2$  or  $\beta$ -actin, and 0.5  $\mu$ l *Taq* polymerase (Life Technologies). The reaction mixture was completed with H<sub>2</sub>O to a total volume of 50  $\mu$ l. Samples were placed in a Gene Amp PCR system 9600 (Cetus, Perkin-Elmer, Norwalk, CT) for 25 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min followed by a 10-min extension at 72°C. After amplification, 15  $\mu$ l of each sample was electrophoresed on a 1.5% agarose gel (Life Technologies). The bands were visualized with ethidium bromide. For quantification purposes, 2.5  $\mu$ Ci of [<sup>32</sup>P]dCTP (DuPont/NEN, Mississauga, ON, Canada) was added to the reaction mixture before PCR. The gels were dried and the bands were analyzed using a phosphorimaging and Image Quant software (Molecular Dynamics, Sunny Valley, CA).

### MLR

A total of 10<sup>5</sup> T cells was cocultured with either 2  $\times$  10<sup>4</sup> or 5  $\times$  10<sup>4</sup> allogeneic APC. For proliferation and cytokine assays, primary MLRs were conducted in 96-well plates. After 5 days, unless indicated otherwise, 1  $\mu$ Ci [<sup>3</sup>H]thymidine was added to the wells for 5 h. The cells were harvested and thymidine uptake was determined using a beta-scintillation counter. Culture medium was recovered from sister cultures to determine the cytokine concentrations. For secondary MLRs, T cells were recovered from the primary MLRs and cocultured with freshly isolated allogeneic APC from the same donor as in the primary MLR. Secondary MLRs were conducted for 3 more days at which time cytokine release was measured.

### L929 cytotoxicity assay

L929 cells (10<sup>4</sup>) were cultured in RPMI 1680 medium with 10% FCS. We added either 50 or 100 U/ml (1.7 or 0.85 ng/ml) of TNF- $\alpha$  in the presence of different concentrations of TNFR:Fc (Genzyme, Cambridge, MA) or carrier buffer. The cells were incubated for 24 h and supernatants were analyzed for lactate dehydrogenase (LDH) content as previously described (24).

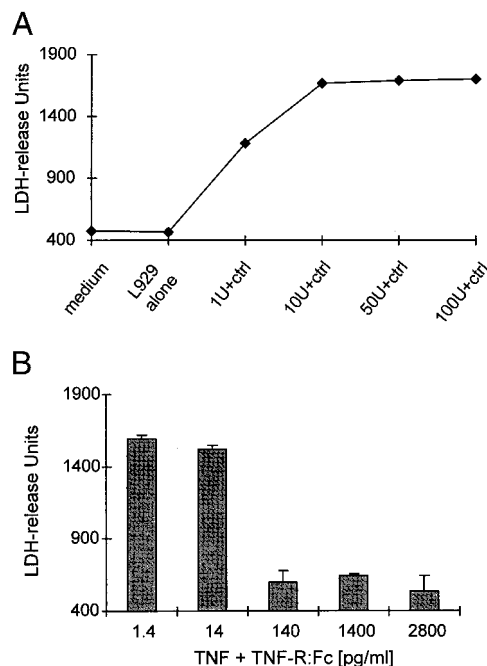
### Cytokine ELISA

IL-12 ELISA kits were obtained from R&D Systems. IL-2, IL-10, and IFN- $\gamma$  ELISA kits were obtained from BioSource International (Camarillo, CA). Tissue culture supernatants were stored at -80°C until analysis. ELISA assays were performed following manufacturers instructions.

## Results

### TNFR:Fc inhibits TNF- $\alpha$ -mediated cytotoxicity

In initial studies, we established that TNFR:Fc could specifically inhibit the cytotoxic effect of TNF- $\alpha$  by exposing TNF-sensitive



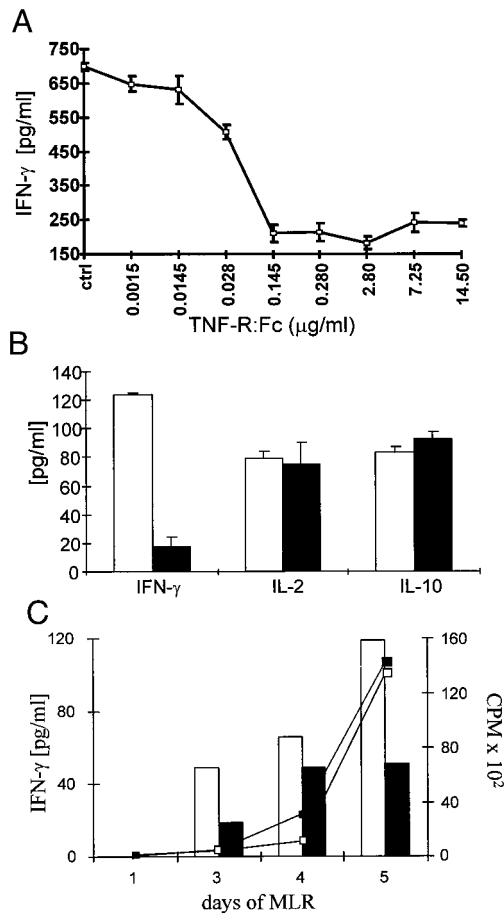
**FIGURE 1.** Inhibition of TNF- $\alpha$  mediated toxicity by TNFR:Fc. **A**, L929 cells 10<sup>4</sup> were cultured for 24 h in the presence of increasing concentrations of TNF- $\alpha$ . Cytotoxicity was assessed by measuring LDH-release. **B**, TNF- $\alpha$  (50 and 100 U/ml) were used to determine effectiveness of increasing concentrations of TNFR:Fc. ctrl = 4  $\mu$ g/ml carrier buffer with IgG1 isotype control.

L929 cells to TNF- $\alpha$  and TNFR:Fc or carrier control. Fig. 1A shows the toxicity of different concentrations of rTNF- $\alpha$  on L929 cells as assessed by LDH release assay. In a subsequent experiment, we have used 50 or 100 U (0.85 or 1.7 ng/ml) of TNF- $\alpha$  that results in maximum LDH release. We can block cytotoxicity mediated by 100 U of recombinant TNF- $\alpha$  using 140  $\mu$ g/ml of TNFR:Fc, indicating that engagement of membrane TNFR on L929 cells is completely ablated by addition of TNFR:Fc (Fig. 1B).

### TNFR:Fc inhibits IFN- $\gamma$ production in an allogeneic MLR

To analyze the effect of TNFR:Fc on immunoregulatory functions, we have performed MLRs in the presence of TNFR:Fc. CD4<sup>+</sup> T cells were isolated from healthy donors and mixed with allogeneic APC at different ratios (5:1–2:1) in 96-well plates. TNFR:Fc or irrelevant IgG1 mAb was added to the reaction. The MLR was conducted for 5 days, and T cell proliferation was assessed by [<sup>3</sup>H]thymidine uptake (23). Supernatants from sister cultures were harvested and analyzed for IFN- $\gamma$  by ELISA.

As shown in Fig. 2A, addition of TNFR:Fc to the primary MLRs inhibited IFN- $\gamma$  production in a dose-dependent fashion. Maximum inhibition was first observed with 140  $\mu$ g/ml of TNFR:Fc. For subsequent experiments we used 2.8  $\mu$ g/ml of TNFR:Fc and 4  $\mu$ g/ml of irrelevant IgG1 in vehicle buffer (ctrl). After 5 days, TNFR:Fc inhibited IFN- $\gamma$  production by 76  $\pm$  7% SEM, on average (Table I). As shown in Fig. 2B, levels of IL-2 and IL-10 were not altered by TNFR:Fc. We did not detect IL-4 or IL-5 under any culture condition used. In time course studies, we observed that TNFR:Fc inhibits IFN- $\gamma$  production from the earliest time point this cytokine could be detected (Fig. 2C). TNFR:Fc does not alter proliferation at the concentrations used over the 5-day time period of the primary MLR.



**FIGURE 2.** TNFR:Fc selectively inhibits IFN- $\gamma$  secretion by T cells. *A*, five-day MLR study in which CD4<sup>+</sup> T cells were mixed with allogeneic APC and increasing concentrations of TNFR:Fc or 4  $\mu$ g/ml irrelevant IgG1 mAb in carrier buffer (ctrl). Culture media were harvested from triplicate wells and individually analyzed for IFN- $\gamma$  by ELISA. Data are expressed as IFN- $\gamma$  (pg/ml)  $\pm$  SEM. *B*, MLR was conducted for 5 days in the presence of 2.8  $\mu$ g/ml TNFR:Fc (■) or 4  $\mu$ g/ml irrelevant IgG1 isotype control in carrier buffer (□). Supernatants were analyzed for IFN- $\gamma$ , IL-2, and IL-10 by ELISA. The mean value  $\pm$  SEM was determined for two individual experiments. *C*, Representative time course experiment showing proliferation and IFN- $\gamma$  secretion determined at different time points during the 5 day primary MLR. There was no difference between the treated and nontreated cultures with regards to cell recovery.

#### APC-dependent TNFR:Fc-mediated inhibition of IFN- $\gamma$ production

Because TNF can be produced by either APCs or T cells, and both cell types are responsive to TNF, subsequent studies were designed

**Table I.** Inhibition of IFN- $\gamma$  production by TNFR:Fc<sup>a</sup>

	IFN- $\gamma$ Production (pg/ml)				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
MLR + ctrl	171	399	284	>1000	563
MLR + TNFR:Fc	23	134	72	296	93
% decrease	87	66	75	70	83

<sup>a</sup> Data indicate results of five individual experiments in which an MLR was carried out for 5 days in the presence of 2.8  $\mu$ g/ml TNFR:Fc or 4  $\mu$ g/ml irrelevant IgG1 isotype control in carrier buffer (ctrl). For each data point, culture medium from triplicate wells was pooled and analyzed for IFN- $\gamma$  by ELISA. Supernatants from APCs alone were used as background control. (% decrease =  $100 \times (1 - [\text{IFN-}\gamma]_{\text{TNFR:Fc}} / [\text{IFN-}\gamma]_{\text{ctrl}})$ ).

**Table II.** Inhibition of APC-derived IL-12 in allogeneic MLR by TNFR:Fc<sup>a</sup>

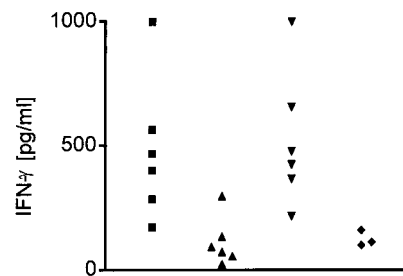
	Expt. 1	Expt. 2	Expt. 3
Ctrl	12	14	81
TNFR:Fc	1	4	26
% inhibition	93	71	68

<sup>a</sup> Data indicate the results of three individual experiments in which an MLR was carried out in the presence of 1  $\mu$ g/ml anti-CD3 for 24 h. A total of 2.8  $\mu$ g/ml TNFR:Fc or 4  $\mu$ g/ml isotype control in carrier buffer (ctrl) were added. For each data point, culture media from triplicate wells were pooled and analyzed for IL-12 p70 by ELISA. The results are expressed in pg/ml IL-12 p70 (inhibition =  $100 - ([\text{IL-12}]_{\text{TNFR:Fc}} / [\text{IL-12}]_{\text{ctrl}} \times 100)$ ).

to determine whether TNFR:Fc-mediated inhibition of IFN- $\gamma$  production involved either an APC-dependent network and/or a direct effect on the T cells. Regarding the former, we have previously shown that TNFR:Fc can inhibit IL-12 production by activated-human adult microglial cells (18). When microglial cells are activated with LPS, they produce TNF- $\alpha$  before IL-12. When we then block the action of autocrine TNF- $\alpha$  by using TNFR:Fc, we could significantly inhibit IL-12 production. In the current study, the levels of IL-12 p70 production in the MLR were at the lower levels of detectability (7.8 pg/ml). To overcome this limitation and to directly determine whether IL-12 p70 production is dependent on TNF, we added anti-CD3 mAbs (0.1  $\mu$ g/ml) to the MLR to activate nonalloresponsive T cells, which in turn results in more robust IL-12 levels. Table II shows the decrease in IL-12 p70 production by APC in MLRs treated with TNFR:Fc measured by ELISA for IL-12 p70.

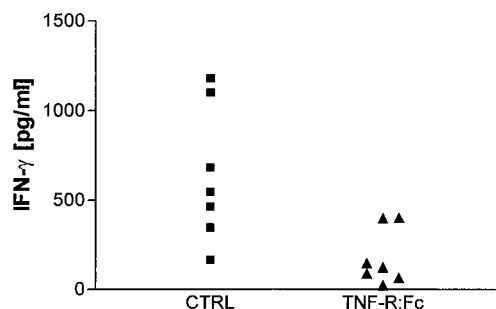
We then went on to establish whether the decrease in IL-12 production by TNFR:Fc-treated APCs is responsible for the decrease of IFN- $\gamma$  in an allogeneic MLR. We added human rIL-12 (R&D Systems) to the cells, to determine whether IFN- $\gamma$  production can be recovered. When 100 pg of IL-12 was added to the cultures, we were able to completely recover IFN- $\gamma$  production by the T cells (Fig. 3). Addition of IL-1 $\beta$  did not rescue IFN- $\gamma$  secretion (data not shown). We could also mimic the effect of TNFR:Fc and decrease IFN- $\gamma$  production by the addition of 0.5  $\mu$ g/ml anti-IL-12 mAbs (Fig. 3).

To establish whether the depletion of TNF during the primary stimulation of alloreactive T cells influences their ability to secrete IFN- $\gamma$  during a secondary MLR in the absence of the antagonist, we isolated and extensively washed the T cells after the initial 5-day primary MLR. These cells were then incubated with fresh allogeneic APC for 3 more days, at which time the culture medium was collected and analyzed for IFN- $\gamma$ . Fig. 4 shows that T cells



**FIGURE 3.** Addition of rIL-12 recovers IFN- $\gamma$  production in TNFR:Fc-treated cultures. MLR was conducted for 5 days. The culture medium from triplicate wells was pooled and analyzed for IFN- $\gamma$  by ELISA. Each data point indicates individual MLRs treated with either 4  $\mu$ g/ml IgG1 isotype control in carrier buffer (■) or 2.8  $\mu$ g/ml TNFR:Fc (▲) or TNFR:Fc + IL-12 (100 pg/ml) (▼) or  $\alpha$ -IL-12 mAbs (0.5  $\mu$ g/ml) (◆).





**FIGURE 4.** MLR was conducted for 5 days in the presence of 2.8  $\mu\text{g/ml}$  TNFR:Fc ( $\blacktriangle$ ) or 4  $\mu\text{g/ml}$  irrelevant IgG1 isotype control in carrier buffer ( $\blacksquare$ ). The nonadherent cells (T cells) were then harvested, extensively washed, and cocultured with freshly isolated APC under regular culture conditions. After 3 days of secondary stimulation, the culture media from triplicate wells were pooled and analyzed for IFN- $\gamma$  by ELISA. Each data point represents an individual MLR.

isolated from TNFR:Fc-treated MLR cultures produce significantly less IFN- $\gamma$  than control cultures when restimulated by fresh APCs under regular culture conditions. As expected, the levels of IFN- $\gamma$  were generally higher in the secondary response. The levels of IL-2 in secondary MLRs were also increased compared with primary MLR cultures but not altered due to the depletion of TNF in the primary MLR (data not shown).

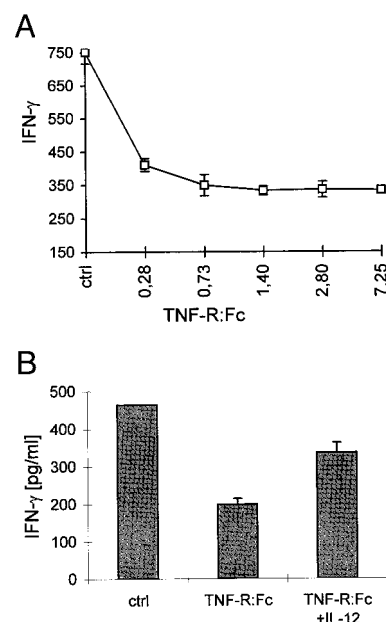
#### Direct TNFR:Fc effects on polyclonally activated T cells

To assess the direct effects of TNFR:Fc on T cells in the absence of APC, we stimulated CD4 T cells for 24 h by polyclonal activation with anti-CD3 mAbs in the presence of TNFR:Fc. Fig. 5A shows inhibition of IFN- $\gamma$  production by activated T cells. The inhibitory effect is less pronounced than in MLRs. Addition of TNF- $\alpha$  to these cultures restores normal IFN- $\gamma$  levels, indicating specificity of TNFR:Fc. In contrast to the MLR studies, we could not mimic the effect of TNFR:Fc by the use of anti-IL-12 mAbs (not shown). However, when recombinant IL-12 was added, we could again increase IFN- $\gamma$  levels (Fig. 5B). These results demonstrate that exogenous IL-12 can override the inhibitory effect of TNFR:Fc on IFN- $\gamma$  production.

As mentioned above, in addition to polarized cytokine profiles, Th1 cells can also be distinguished from Th2 cells by other phenotypic markers. During the course of Th2 polarization induced by anti-IL-12 and IL-4 treatment in vitro, the Th0 and Th1 cells down-regulate IL-12R  $\beta_2$  subunit and display a decreased responsiveness to IL-12. Treatment with IFN- $\gamma$  or IL-12 can restore higher levels of this receptor (14). After stimulation for 24 h with anti-CD3, we performed a radiolabeled semiquantitative PCR for IL-12R  $\beta_2$ . The gel was analyzed by phosphorimaging. Fig. 6 shows that anti-CD3-activated T cells in TNFR:Fc-treated cultures express significantly less IL-12R  $\beta_2$  message than control cultures, indicating that TNF- $\alpha$  can mimic some of the functions ascribed to IFN- $\gamma$ .

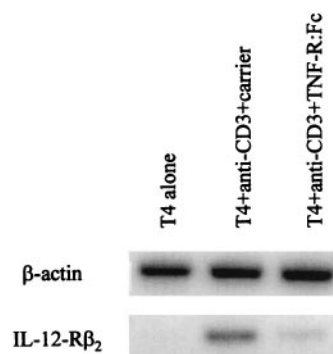
## Discussion

Our study delineates a cytokine network involving TNF/LT and IL-12 that regulates the polarization of Th1 T cells. We show that by inhibiting the action of APC- and T cell- derived TNF/LT in an MLR, one also decreases the production of IFN- $\gamma$  by CD4 T cells. TNFR:Fc does not alter IL-2 or IL-10 levels. This finding supports our conclusion that treatment with TNFR:Fc selectively inhibits IFN- $\gamma$  secretion without impacting on general lymphocyte function and survival or driving Th2 polarization. We have previously



**FIGURE 5.** Dose-dependent inhibition of IFN- $\gamma$  production in polyclonally activated pure CD4 T cells. *A*, CD4 T cells were activated with 1  $\mu\text{g/ml}$  anti-CD3 mAbs in the presence of increasing concentrations of TNFR:Fc or 4  $\mu\text{g/ml}$  irrelevant IgG1 isotype control in carrier buffer (ctrl) for 24 h. Culture media were pooled from triplicate wells and analyzed for IFN- $\gamma$  production by ELISA. Results are expressed as the mean of two experiments  $\pm$  SEM. *B*, Activated-CD4 T cells were treated with 2.8  $\mu\text{g/ml}$  TNFR:Fc in the presence or absence of 100 pg/ml IL-12 p70 or 4  $\mu\text{g/ml}$  ml irrelevant IgG1 isotype control in carrier buffer (ctrl) for 24 h. Culture media were pooled from triplicate wells and analyzed for IFN- $\gamma$  production by ELISA. Results are expressed as the mean of two experiments  $\pm$  SEM.

shown that TNF- $\alpha$  and IFN- $\gamma$  are important costimuli for the induction of IL-12 expression in APC (18). IL-12 is the most potent soluble factor driving the development of a Th1 profile by T cells (16, 25). Here, we demonstrate a significant reduction of IL-12 p70 production by APCs in allogeneic MLRs supplemented with TNFR:Fc. Addition of rIL-12 to the TNFR:Fc-treated cultures restores normal IFN- $\gamma$  levels, indicating that the dominant effect of TNFR:Fc on this system is the inhibition of IL-12 production by APCs.



**FIGURE 6.** TNFR:Fc decreases IL-12R $\beta_2$  subunit expression in activated-CD4 $^+$  T cells. CD4 T cells were activated with 1  $\mu\text{g/ml}$  anti-CD3 mAbs in the presence of either TNFR:Fc (2.8  $\mu\text{g/ml}$ ) or IgG1 isotype control (4  $\mu\text{g/ml}$ ) for 24 h. Background control is derived from nonactivated T cells. A representative autoradiograph from a 25-cycle PCR for IL-12R  $\beta_2$  (281 bp) and  $\beta$ -actin (378 bp) is shown.

The APC-dependent mechanism may not be the only modulator of IFN- $\gamma$  production by T cells. We show that in the absence of APCs, polyclonally activated pure CD4<sup>+</sup> T cells decrease IFN- $\gamma$  production when treated with TNFR:Fc. The effect is less pronounced than in APC-stimulated T cells. It is feasible that TNF acts directly on T cells to maintain a Th1 phenotype by enhancing IL-12 responsiveness (via IL-12-R $\beta$ 2) and up-regulating IFN- $\gamma$  production. The direct inhibitory effect of TNFR:Fc on T cells can be overridden by exogenous IL-12. The latter finding supports the hypothesis, that the inhibition of IFN- $\gamma$  production by TNFR:Fc is predominantly achieved via its effect on APC, in particular, IL-12 production. Ultimately, IFN- $\gamma$  itself can also contribute to this cytokine network in a feedback fashion by further stimulating IL-12 production in activated APC.

TNFR:Fc has now been used therapeutically both in experimental and human autoimmune inflammatory disorders. TNF levels produced by monocytes in MS are increased and correlate with disease severity (26). Animals treated with TNFR:Fc after immunization with myelin basic protein or proteolipid protein but before onset of clinical symptoms do not develop disease but have continued inflammation as assessed by the number of CNS infiltrating leukocytes (19). The authors concluded that the therapeutic effect of TNFR:Fc reflected blocking the effector functions of TNF. TNF<sup>-/-</sup> mice also display a delayed onset of clinical symptoms (29), an effect that could be explained by either regulatory or effector functions. Our data indicate that it is also possible that treatment with TNFR:Fc may alter the cytokine polarization of the infiltrates by lowering the levels of IL-12 and subsequently IFN- $\gamma$ . Reduced IFN- $\gamma$  production would result in reduced activation of bystander cells such as macrophages and microglia. Patients with MS also are reported to have elevated cerebrospinal fluid and serum IL-12 levels (27, 28).

Our in vitro study provides insight as to how TNFR:Fc can influence cytokine networks that regulate the polarization of cytokine patterns. Immune therapy in RA with TNFR:Fc has been shown to be efficacious for clinical symptoms. However, when patients with MS were treated with TNFR p50-IgG fusion protein, there was a reported increase in the relapse rate lesion formation as assessed by magnetic resonance imaging (22). Neither study provided data on whether there was skewing of the T cell cytokine response, in a manner demonstrated in our study. To understand the in vivo effects of TNF/LT-directed therapy, one needs to consider the complicated pharmacokinetics and the pleiotrophic nature of the cytokines TNF and LT. Our current study may provide an approach to determine that desired in vivo effects are occurring and minimize the risk of clinical toxicity.

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